INTERACTION BETWEEN ANTIGENIC COMPETITION AND THE GRAFT-VERSUS-HOST REACTION

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by

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Dedicated to my Parents
and my Grandparents

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I. Introduction

Tolerance to sheep red blood cells (SRBC) in hybrid rats can be abrogated by initiating a graft-versus-host reaction in the tolerant animals, using lymphocytes from one of the parental strains as the graft (34). At least three observations suggest that this effect is dependent upon antigenic stimulation of the exogenous lymphocytes by host tissue, rather than upon the mere introduction of immunocompetent cells: 1) Parental lymphocytes from donors which have been made tolerant to the histocompatibility antigens of the opposite parent (but which are still responsive to SRBC) are incapable of eliminating SRBC tolerance in F_1 hybrids. Yet, parental cells which are tolerant to SRBC but not to the host can still restore the SRBC response. 2) The cells which produce anti-SRBC antibody following elimination of tolerance are largely of host, rather than of donor, origin. 3) The number of antibody-forming cells produced seems to be unrelated to the number of allogeneic cells injected, over a dose range of $3 \times 10^8 - 11.5 \times 10^8$ cells.

These findings suggested to McCullagh that the abrogation of tolerance in this situation was not based upon the creation of new antigen-reactive cells, but rather upon the de-repression of cells which pre-existed in the tolerant host and which were vital to, but temporarily unable to par-

ticipate in, the humoral antibody response to SRBC.

Mechanisms of this type, based upon an endogenous form of active immunosuppression, have been postulated not only for tolerance, but also with respect to other phenomena involving immunological unresponsiveness. One of these, antigenic competition, is the subject of the present study. Competition, as defined by Adler (1), refers essentially to the partial or complete inhibition of the immune response to a particular antigen as a result of the administration of another antigen. Since an actively suppressive component has been implicated in both tolerance and competition, and since a graft-versus-host reaction (GVHR) was shown to restore specific immunologic reactivity in tolerant animals presumably on the basis of de-repression, the question asked in the present study is whether a GVHR is also capable of negating the effects of antigenic competition.

Before presenting the findings, some consideration will first be given to a number of aspects of the immune response in general, as they may relate to the topic currently under investigation. Following this, there will be a discussion of some of the earlier and more recent studies which have brought us to our present level of understanding and speculation with regard to antigenic competition. Through a selective emphasis of particular concepts, the present experiment may hopefully be placed into better perspective.

II. T-Cells and B-Cells

The primary reservoirs of lymphoid tissue--lymph nodes, thymus, and spleen--are inhabited by cells whose ultimate precursors may be traced to the bone marrow (36). Through labelling techniques, and comparison of high mitotic rates with low cell death rates within the thymus, there is now fairly good evidence that some (but not all) of the lymphoid cells emigrating from the marrow reside briefly in the thymus before going on to populate the spleen and nodes (36, 38, 40). Migration from the thymus seems to be organ-specific for the components of the peripheral lymphoid system (59).

Not inconsistent with these observations is the extensive role which the thymus plays in the immunoreactivity of animals (primarily mammals) known to possess one. Thymectomy in the mouse within twenty-four hours of birth produces a lymphopenia, together with a markedly impaired ability to reject a homograft, exhibit delayed hypersensitivity, or produce a humoral antibody response (38, 43, 49). Subcutaneous implantation of whole, intact, neonatal thymus within several days of thymectomy can reverse all of these deficiencies, in contrast to an intravenous injection of neonatal spleen cells, which has virtually no restorative effect (48, 49).

A related phenomenon may be demonstrated in adult mice rendered immunologically incompetent by a lethal dose of

X-irradiation. Such mice may be reconstituted immunologically and saved from death by the administration of a syngeneic bone marrow cell graft. However, if thymectomy is performed prior to injection of the graft, immune responsiveness does not return (40).

Because thymectomy does not immediately produce the detrimental effect in an adult mouse which is seen in the neonate, there has been some speculation that the thymus ceases to be functional shortly after the neonatal period. Further studies, however, reveal that the onset of immuno-incompetence is merely delayed in mature animals, appearing six to nine months after thymectomy (39). This has contributed to the alternate notion that the thymus is continually maintaining and replenishing a pool of "specially educated" lymphocytes which participate in some vital way in the immune response. In the absence of the thymus, presumably, the pool is gradually depleted. While this may happen rather quickly in the neonate, in whom a substantial pool has not yet had time to be established, thymectomized adults may go for a number of months before exhausting their supply of thymus-processed cells (38, 39, 41).

As was noted previously, a brief residence in the thymus is characteristic of only some of the lymphoid cells in the body. Those cells which pass through the thymus and those which do not are now believed to constitute two distinct populations, each with its own role in the immunologic process.

In support of this is the fact that the humoral antibody response in animals lethally irradiated and reconstituted with either bone marrow cells or thymocytes (but not with both) is only a small fraction of the response seen in animals reconstituted with marrow and thymus together. The effect of combining the two cell types is not merely additive but is synergistic, suggesting that an interaction occurs between these two populations (7, 8). The suggestion is strengthened by the observation that lymphoid cells obtained from a neonatally thymectomized mouse are still capable of giving rise to antibody-secreting cells in the presence of thymocytes and an antigenic stimulus (41, 42).

Also of great significance is the fact that thymocytes themselves appear to be incapable of producing antibody or of being transformed into cells which do (11, 44). Yet, through chromosomal labelling techniques, and more recently through incorporation of I^{125}-labelled 5-iodo 2-deoxyuridine (IUDR) into lymphoid tissue, thymus-derived lymphocytes have been shown to undergo a wave of mitosis following antigenic stimulation (11, 20, 42, 44).

These observations have contributed to the current concept of the immune response, in which the first step is an interaction between the antigen and thymus-derived lymphocytes (T-cells). The function of the T-cell is somehow to effect the transformation of precursors of antibody-forming cells (B-cells) into cells which secrete an antibody specific for the antigen (42, 44). The B-cell is not descended from a

thymus-processed lymphocyte, but is rather a member of the cell population which bypasses the thymus after emerging from the bone marrow (44).

With a two-cell notion of the immune system thus established, attention may now be paid to the events, both real and speculative, which underlie a particular immunological phenomenon.

III. Antigenic Competition: A Description and Some Proposed Mechanisms

As mentioned on page 2, antigenic competition refers to a depression of the immune response to an antigen, brought about by the administration of another antigen. The phenomenon is not confined to the humoral antibody system, since antigen administration can be shown to prolong the survival of a subsequently placed homograft (15), and homograft rejection is largely on a cellular basis. The degree of inhibition observed, and the time interval which must be allowed between administration of the two antigens in order to produce the maximum effect, are both variable, depending upon such parameters as dosage; antigen combination used; the type of animal in which the phenomenon is being observed; and who is performing the study.

Many mechanisms have been suggested to explain the occurrence of competition. A number of these are based upon the <u>clonal selection</u> theory of acquired immunity, the main proponents of which have been Burnet and Lederberg. Essentially, the theory (6, 54) presupposes the existence of cellular units, each having been rendered genetically capable of synthesizing an antibody specific for a single antigen. The number of units capable of producing the same antibody is small prior to contact with the associated antigen. The number of dif-

ferent types of units, with respect to the type of antibody produced, is large -- presumably because of a high rate of somatic mutation among antibody-forming cells. All cells descended from the same precursor are collectively referred to as a clone.

The antigen "selects" for its corresponding clone by combining with antibody specific for itself on the surface of cells producing that antibody. Pre-existence of the antibody is ascribed to a minute amount of ongoing synthesis by all cellular units, independent of contact with antigen. The antigen-antibody reaction on the cell surface serves as a stimulus for proliferation, resulting in a substantial increase in the number of cells producing that particular antibody. Thus, the antigen itself becomes the agent which "selects" for the growth and predominance of specific "clones" of immunological cell units.

An alternative to this view is the "instructional" hypothesis (25), which assumes that all immunocompetent cells are equivalent prior to antigenic stimulation. Contact between an antigen and any immunological cell results in the initiation, rather than augmentation, of specific antibody synthesis. Presumably, the antigen acts as a template, or as the inducer of a template, for protein synthesis. Thus, specificity is an acquired, rather than an a priori, characteristic of immunologically reactive cells, and appears only as a result of contact with a particular antigen.

Theories of competition may be based upon one or the

other of these mechanisms, or in some cases may be suited to either. The following is intended as a sample, but certainly not an exhaustive review, of ideas which have been offered to explain antigenic competition. These are presented together with observations by other workers who have viewed these hypotheses as somewhat less than adequate.

The clonal selection theory readily lends itself to an explanation of competition if one assumes that the stimulation and subsequent growth of particular clones will interfere with the growth of other clones, on either a spatial or a nutritional basis (1, 24). Invoking spatial limitations is not valid, according to Schechter (52), in view of the results obtained when the same antigen is used in an effectively competing and non-effectively competing combination. Assume, for example, that A and B are mutually competitive antigens -- that is, the antibody response to A when administered together with B is less than that seen when A is given alone, and a similar relation holds for B. Assume further that A and C are non-competitive, so that the response to either is unaffected when the two are administered together. Since the anti-A response is greater in the A-C combination than in the A-B, we would expect, on the basis of spatial limitations, to find the anti-C response more impoverished than the anti-B. As mentioned above, however, the opposite is true.

Competition between clones for cellular nutrients is argued against by Cremer (10), who demonstrated that the amount

of an antibody produced by cells in culture was independent of whether or not there were other cells in the culture concurrently producing a different antibody.

Competition at the level of the antibody-forming cell has been invoked by others in a manner more consistent with the "instructional" than with the clonal selection hypothesis. In this situation, one assumes that most of the cells currently capable of producing antibody are brought into play by the administration of the first antigen, so that fewer of them remain to be recruited by the second. If both antigens are given simultaneously, then the antibody-forming cells must be "shared" between them, with a resultant impairment in the response to each.

There are a number of further assumptions which must underlie this view. One is that most cells are capable of producing no more than one antibody at a time (1, 35). Another is needed to explain the fact that for certain antigen combinations, simultaneous administration results in a diminished response to only one of the antigens, while the response to the other remains intact. Schechter (52) seems to beg this question when he postulates that such cases represent an unexplained preference somewhere in the immunological system for the determinants carried by one of the antigens.

Waterston (58) rejects the notion of competition at the level of the antibody-forming cell by citing a paradox. He notes that when two antigens are administered consecutively in vivo, an interval of four days between administrations gives

a maximum competition effect. Yet, when the first antigen is given in vivo and the second in vitro, using a culture of spleen cells taken from the original animal, a four-day interval produces the <u>least</u> amount of competition. Furthermore, when Ag-A is added to a culture of spleen cells taken from mice immunized with Ag-B, the anti-A response is better than when the cells are taken from normal mice. These findings argue against exhaustion of an antibody-forming cell population as the source of competition.

Perhaps the strongest evidence against competition for an antibody-forming cell is the fact that antigenic competition is demonstrable even in the face of total unresponsiveness to the first antigen. This means that the administration of Ag-A to an animal rendered unresponsive to it can result in a poorer response to a subsequently administered dose of Ag-B than when B alone is given. This was shown by Gershon (23), who used the passive antibody technique to eliminate endogenous antibody production against the preimmunizing antigen. He found that mice preimmunized under these conditions still had a poorer response to a subsequent test antigen than when the latter was given alone.

This finding has been reported by other workers who used the method of induced tolerance, rather than passive antibody, to create unresponsiveness to the first antigen. However, the appearance of competition under these circumstances is difficult to accept for the following reason: Each individual is endowed with certain antigenic constituents of his own body

which normally do not stimulate the production of antibody against themselves. Although exposure to these tolerated antigens is occurring at every instant in time, it would be unreasonable to assume that a continuous state of immunological depression (through antigenic competition) existed by virtue of such exposure. One must therefore conclude, almost by definition, that a fully tolerated antigen cannot also function as a competitive one. Indeed, Liacopoulos (31) was able to demonstrate competition only during the induction of tolerance to the first antigen, but not once tolerance was fully established. One might speculate as to whether the animals studied by Schechter (52) were also in a state of partial, rather than complete, tolerance at the time the experiment was performed.

At any rate, the evidence cited previously suggests that we look somewhere other than at the level of the antibody-making cell in order to explain the suppressive effect of pre-immunization. Waterston (58) theorized that the introduction of an antigen might cause the consumption of some humoral factor in the serum which is permissive for the antibody response. When a second antigen is given shortly after the first, a normal response to it cannot occur, because of the absence of this factor. It has been shown by Gershon (22), however, that the administration of isologous serum does not have a restorative effect on the response to the second antigen.

Evidence that an antigen must first be "processed" by a phagocyte in the reticulo-endothelial system before being

able to stimulate an immunocompetent lymphocyte (56) has led to a theory of competition based on "reticulo-endothelial blockade" (1, 14). In essence, it assumes a limit to the number of phagocytes available for antigen processing in any particular area of the body. Antigens administered simultaneously would compete for these cells, or, if one antigen preceded the other, it would "tie up" or block the R-E system, so that a reduced number of processing cells would be available when the second antigen was introduced.

Those who are dissatisfied with this view call attention to the fact that when the two antigens are injected into different sites, with considerable spatial separation between them (contralateral foot pads, for example, or intravenous vs. intraperitoneal), competition may be equally or even more marked than when the two antigens are injected at the same site (1, 15, 16). Since a different population of phagocytes is presumably involved in the processing of each antigen when separate sites are used, one would not expect as significant a degree of competition to occur under those circumstances if R-E blockade were the underlying mechanism. The issue does seem to be clouded, however, by other studies which claim just the opposite result -- an inability to produce competition <u>except</u> when both antigens are injected into the same site (5, 47).

Some observers have suggested that competition represents a state of partial tolerance to the second antigen. The assumption in this case, as pointed out by Eidinger (16), is that the two antigens share some determinants in common.

Their sequential administration, then, has the same effect as a single high-dose of an antigen with those common determinants. The effect is tolerance, at least on a partial basis, with specificity for those particular characteristics.

Such a sharing of attributes is unlikely, according to Eidinger, in view of the fact that competition may occur between two essentially non-cross-reacting antigens. Furthermore, in partial tolerance, the affinity of the antibody for the antigen appears to be decreased (55), while in Eidinger's competition experiments, affinity for the second antigen was unaltered.

A similar principle underlies the so-called "feedback" theories of competition, such as that described by Uhr (57). Like the partial tolerance view, such a theory assumes the existence of some common determinants between the first and second antigen. The antibody produced against the first then interacts with the second and depresses the response to it, acting in much the same way as passive antibody against the second antigen.

This interpretation is difficult to accept in view of the evidence that antibody synthesis against the first antigen need not occur in order for competition to be present (23). It is also undermined by the ability of non-cross-reacting antigens to compete effectively.

With the evolution of the T- and B-cell concept, and the implication that cells which primarily interact with antigen are distinct from those which become antibody-producers, it

became possible to postulate competition at a cellular level using T-cells as the exhaustible population (2). This view is considered by Möller and Sjöberg (47), who reject it on the basis of evidence supplied by Radovich and Talmage (51). These workers, using lethally irradiated mice, showed that competition was stronger in animals who had been reconstituted with the largest number of spleen cells -- just the opposite of what would be expected if competition were due to a limitation in the number of antigen-reactive cells available. Gershon (22) demonstrated this result in a manner more specific for the T-cell by using thymectomized mice which were then reconstituted with varying numbers of thymocytes. The greatest amount of competition was elicited in those animals receiving the largest number of T-cells.

Further work by these and other investigators has led to the suggestion that antigenic competition does not really involve competition for anything at all, but rather represents the creation of an internal environment which is actively unfavorable at some level to the production of antibody in response to antigen. One way such an environment could come about is through the elaboration of a local or humoral immunosuppressive substance, consequent upon the introduction of antigen (22, 47, 51). This is the view which is emphasized in the present study, and which will now be considered in more detail.

IV. The Immunosuppressive Theory of Antigenic Competition

Demonstrating a phenomenon which they called "pre-emption," O'Toole and Davies (50) harvested the lymph nodes draining the sites of subcutaneous injections of antigen in mice. They found that the number of cells in these nodes making antibody against the test antigen (measured as Plaque-Forming Cells, or PFC) was significantly reduced by intraperitoneal administration of another antigen four days previously. This was true even when the first and second antigens were identical, which means that the antigen actually competed with itself. The findings suggested a depression of immune responsiveness in general, rather than the "using up" of a cell or factor in the immunological pool. If the latter were the case, the number of PFC would be expected to increase slightly, or at worst remain constant, following a second dose of the same antigen. A decrease seems most consistent with a generalized suppressive effect.

A humoral basis for the apparent immunosuppression seen in competition is suggested by the work of Möller and Sjöberg (47). These workers produced competition in mice which had been sublethally irradiated following immunization with the first antigen and then inoculated with adoptively transferred spleen cells prior to administration of the second antigen. The response to the second was significantly poorer than in

mice who received no antigen prior to irradiation. This was true even when the transferred spleen cells were from donors which had been sensitized to the test antigen.

Persistence of the competitive effect of an antigen after lethal (rather than sublethal) irradiation and reconstitution has also been demonstrated (22, 58). Gershon noted that two days must be allowed between administration of the first antigen and subsequent irradiation in order for competition to appear. This suggests that the establishment of an immunosuppressive milieu within the animal is a cell-dependent process, which requires several days to reach detectable proportions. O'Toole and Davies (50), using normal mice, removed the spleen at varying intervals following administration of a first antigen. They found that splenectomy within twenty-four hours of immunization destroyed the competitive effect of the first antigen upon a second given four days later. Splenectomy any time after twenty-four hours had no deleterious effect upon competition. These results support the concept of a time- and cell-dependent humoral immunosuppression.

The dependence of competition upon cellular activity has already been alluded to on pages 14 - 15. There it was noted that the degree to which competition can be elicited in an irradiated or thymectomized animal is directly related to the number of spleen or thymus cells which are subsequently restored.

Evidence for cell-mediated unresponsiveness following administration of an antigen is not restricted to antigenic competition, the same effect having been demonstrated for immuno-

logical tolerance. Like competition, tolerance appears to be a thymus-dependent phenomenon (21).

What the exact nature of the immunosuppressive factor might be, beyond its intimate association with the T-cell, is still unclear. The level(s) at which it may operate are also in need of further definition. Möller (45) hypothesized that when sensitized T-cells in culture were exposed to antigen, they released a substance which rendered other lymphocytes incapable of responding to a mitogenic stimulus given three days later. Möller and Sjöberg (47) suggested that the effects of the immunosuppressive factor were aimed at the level of T-cell - B-cell interaction. Thus, T-cells would be rendered incompetent to stimulate B-cells and/or B-cells would become unresponsive to stimulation by T-cells, so that they would not differentiate into antibody-producing units.

Gershon (21) suggested that such a factor -- for which he proposed the name Ig Y -- could act to "turn off" both thymus-derived and bone marrow-derived lymphocytes. In his discussion, he noted that a substance tentatively called Ig X had previously been proposed by Mitchison as a factor which facilitated T - B-cell interactions, and which was presumably elaborated as a result of antigenic stimulation.

Recently, Gershon succeeded in actually demonstrating the existence of the "suppressor T-cell" (19), which can significantly reduce the mitotic response of other T-cells to an antigenic stimulus -- "turn them off," in effect.

On the basis of these findings, immune responsiveness might appropriately be viewed in terms of relative amounts of stimulatory and suppressive substances acting at any one time, and in terms of variations in the balance between these substances over periods of time. The concept will be discussed at greater length in connection with the results of the pre-ent study.

V. Materials and Methods

Mice

The test animals were eight-week-old male C3D2F1 mice (genetically C3H x DBA/2 hybrids). Donors of allogeneic thymocytes were five- to six-week-old male mice of the parental C3H strain. All animals were obtained from Jackson Laboratories, Bar Harbor, Maine.

Antigen

Sheep or horse red blood cells were obtained in Alsever's solution and washed four times with 0.9% saline solution. They were injected intraperitoneally as a 20% cell suspension, in a volume of 0.2 cc.

Thymocyte cell suspension

Thymocyte donors were killed by cervical dislocation, and their thymuses removed and placed in cold Medium 199. Cell suspension was obtained by gently moving the thymuses about between ground glass slides within the medium. The suspension was filtered through three layers of gauze and washed twice with fresh Medium 199. Cell count was then performed using a hemocytometer and the Trypan blue dye exclusion method. Mice that were to undergo a graft-versus-host reaction received 5×10^7 parental thymocytes in a volume of 0.2 cc., injected intravenously via the tail vein.

Bleeding and titration

Mice were bled serially from the retro-orbital plexus. Serum was separated by centrifugation within one hour of bleeding, and titrations were performed within twenty-four hours (the serum being kept refrigerated in the interim). All mice were earmarked, and their antibody titers followed separately.

Titration was performed by the microhemagglutination method, with titers expressed as the $\log_2$ of the highest dilution of serum still showing grossly observable agglutination. For example, a titer of 3 would mean that agglutination was observable when the serum was diluted $1:2^3$ (or 1:8) with normal saline, but not when it was diluted $1:2^4$ (or 1:16). If agglutination was observable only in the undiluted serum, a titer of zero was recorded. If no agglutination at all was observed, the titer was recorded as $\bar{1}$, and was assigned a value of -1 in all statistical computations.

Following the initial antibody determinations, the sides of the plates were tapped gently until the red cells were resuspended, and 0.025 ml. of 0.1M 2-mercaptoethanol (2-ME) was then added to each well. After standing for two hours at room temperature, the plates were again read. These titers were now assumed to represent the level of mercaptoethanol-resistant antibody, which is roughly equivalent to 7s antibody (12). This resuspension technique has been found to yield results equivalent to those which would be obtained by using 2-ME at the outset in a separate titration (53).

VI. Experimental Design

The variables studied were antigenic competition (present or absent) and GVHR (present or absent). This yielded the four groups shown in Fig. 1. At least four animals were present in each group, the largest number in any group being seven.

All mice received SRBC (the test antigen) on day 0. Those selected to undergo antigenic competition received HRBC four days prior to this (day -4). The cross-reactivity between these two antigens is considered to be negligible (47). An interval of four days between H- and SRBC was used because it has been shown to produce the optimum amount of competition for that combination of antigens. When longer or shorter intervals are used, the response to the test antigen is not as markedly depressed (50, 51, 58).

A GVHR was initiated in the appropriate animals on day 0, preceding by no more than two hours the injection of the test antigen. Humoral antibody titers were measured on days 0 (baseline), 4, 8, 15, and 22. All animals were titrated for antibody against HRBC as well, in order to ascertain that this antibody was indeed being produced in the groups selected to undergo competition.

VII. Results

Because the endpoints of agglutination were often highly equivocal prior to treatment with 2-ME, only the titers of ME-resistant antibody are presented here. This represents the approximate level of 7s antibody, which appears after about the second post-immunization day and persists for a much longer period of time than does the ME-sensitive 19s antibody (27).

The experiment was actually performed four times. The mean SRBC antibody titer for each group on each of the observation days is listed in Table I for all four experiments.

A. Effect of GVHR upon antigenic competition

In order to quantitatively compare the amount of competition occurring in normal mice with that occurring in mice undergoing a GVHR, an Index of Competition was created, according to the following formula:

$$I.C. = \frac{T_{comp} + 2}{T_{cont} + 2} \quad , \text{ where}$$

T_{comp} = Mean anti-SRBC titer in a group preimmunized with HRBC.

T_{cont} = Mean anti-SRBC titer in the appropriate control group.

A factor of 2 was added to numerator and denominator to avoid the potential problem of division by zero, since the lowest

possible mean titer in any group was -1. A formula similar to this but without the constant term was used by Dukor and Dietrich (14) to express the magnitude of antigenic competition.

In the present situation, Group 2 serves as a control for Group 1 (neither of these groups undergoing a GVHR), and Group 4 serves as a control for Group 3 (both of these undergoing GVHR). As competition becomes more pronounced, the value of the I.C. decreases. Conversely, as the response in a preimmunized group becomes more nearly equal to that of the non-preimmunized control, the I.C. approaches 1. An I.C. greater than 1 indicates a reversal of the expected competition effect -- that is, a response of greater magnitude in the preimmunized group than in the respective control. The results appear in Fig. 2.

For reasons which were not entirely clear, competition was often difficult to maintain in normal animals (animals not undergoing GVHR). It appears, however, that the <u>early effect</u> of a GVHR in each experiment was to <u>enhance any antigenic competition which was occurring</u>. Only in Expt's. 2 and 4 did the enhancement persist through the last observation day, becoming virtually negligible in Expt. 4. In the remaining cases, the effect had transformed by day 8 into one which mitigated the degree of competition present, actually reversing it in Expt. 3 (I.C. greater than 1).

B. Effect of antigenic competition upon GVHR

There is another, and perhaps a more profitable, standpoint from which these data may be considered -- that is, in terms of

the effect of antigenic competition upon the dynamics of the graft-versus-host reaction. The question then becomes the following: If the presence of a GVHR has some sort of effect upon the immune response to SRBC in normal mice, how is this effect altered by the simultaneous occurrence of antigenic competition?

To ascertain the effect of a GVHR in normal animals, the SRBC titer of Group 4 was compared with that of Group 2 (see Fig. 1). To determine the effect in preimmunized animals, Group 3 was compared with Group 1. This yielded two differences -- $(\text{Gp. } 3 - \text{Gp. } 1)_{\text{day } n}$ and $(\text{Gp. } 4 - \text{Gp. } 2)_{\text{day } n}$. The effect of competition is then the difference between these differences. This is illustrated graphically in Fig. 3. Each line represents the difference between a GVH group and its respective control. One line in each graph is derived from normal animals and the other from animals undergoing antigenic competition. Since the values in Table I were used for these calculations, the ordinate is expressed as the difference between two logs.

Quite apparent is the fact that a GVHR did not have a consistent effect in normal animals, either from week to week or from experiment to experiment. Of interest, however, is the <u>attenuation or complete reversal of the GVH effect by the presence of competition</u>, which is evident in twelve of the sixteen observations made. In the remaining four instances, an <u>accentuation</u> of GVH effect by competition is noted. These results are summarized in Fig. 4.

Thus, the GVHR sometimes had a stimulatory, and sometimes

a depressive effect on the response to SRBC in normal animals (animals not undergoing competition), and this effect changed over time. But whatever the magnitude or direction of the difference in response caused by GVH, the effect of preimmunization with HRBC was always to modify this difference, usually by attenuating or reversing it.

VIII. Discussion

Some general aspects of the graft-versus-host reaction should first be considered in relation to the present study. Many of these concepts are presented more fully in Simonsen (54).

Successful initiation and maintenance of a pure GVHR requires not only an antigenic histologic difference between host and graft, but also an inability on the part of the host to reject the grafted cells. The latter condition may arise in a number of ways. Neonatal or embryonic hosts, for example, are often incapable of rejecting foreign histocompatibility antigens. Immunocompetent adults, however, will reject virtually all such antigens, tolerating only those with which they themselves have been genetically constituted (the recognition of "self"). Thus, an F_1 hybrid will ideally accept cells from either parental strain, since it is endowed with a genetic contribution from each parent. If the grafted cells are immunocompetent, however, they will recognize as "foreign" the antigenic component of the host which is representative of the opposite parent. The result will be a unidirectional immunological attack launched by the graft against the host. The graft, having been recognized as "self," is not reciprocally attacked, and is thus free to exert its effects unencumbered by any threat of rejection.

This is the model utilized in the present study. The

C3D2F1 mice which were used are hybrids of the strains C3H and DBA/2, which differ genetically at the H-2 locus. Differences at this location are known to be strongly antigenic in terms of histocompatibility, and have thus been used to produce reliable graft-versus-host reactions.

For present purposes, a severe GVHR was deemed undesirable, since the animals might be incapacitated to an excessive degree. Thus, strength as well as reliability of the reaction must also be considered. The violence of a GVHR is determined to some extent by the antigenic strength of the particular genetic difference between donor and host, a factor which must be established on an empirical basis. Also important are the number and type of cells grafted, as well as the age of the recipient. Younger animals tend to undergo more violent reactions. Cell number, as might be expected, is positively correlated with severity. With regard to cell type, the thymus has been shown to produce the mildest GVHR per number of cells grafted, when compared with spleen, lymph node, or thoracic duct lymphocytes (9, 28, 44).

The virulence of a reaction may be assessed grossly on the basis of "classic" manifestations of the GVHR: Growth retardation and emaciation (runting), diarrhea, hepatosplenomegaly, lymphoid atrophy, and anemia. A more quantitative evaluation may be based upon per cent mortality, failure to gain weight, or various parameters involving the weight of the liver or the spleen.

Many of these manifestations are interpreted as "rejection"

phenomena, with the host himself serving as an antigen which is being rejected. This view is consistent with the observation that lymphocytes undergo a mitotic burst shortly after coming into contact with the cells of an allogeneic individual, either in vivo or in vitro (17, 33, 60). This burst is not observed in cells taken from donors made tolerant to the recipient (17). Furthermore, lymphocytes in culture will, in the presence of allogeneic lymphocytes, become cytotoxic for allogeneic fibroblasts. All of this bears an obvious parallel to the mitotic behavior of lymphoid cells following contact with an antigen (page 5).

The effects of a GVHR should not be thought of as uniformly deleterious, since immunological competence of the host may actually appear to be enhanced during mild reactions. This is demonstrated in the present results by the increased response to SRBC which was sometimes seen in normal mice undergoing a GVHR. The effect has also been noted by others (13, 29, 34, 46), but in each case, has been observed only when an interval of three days or less separated the initiation of the reaction and the subsequent administration of the test antigen. The findings of McCullagh (pages 1 - 2, (34)) suggest that allogeneic T-cells are necessary for the stimulatory effect, since tolerance could not be eliminated by the injection of allogeneic bone marrow cells, while spleen, thymus, and thoracic duct lymphocytes were all successful in this regard. Also, the point should be re-emphasized that this increase in the immune response could not be attributed merely to a direct interaction

between the test antigen and the grafted lymphocytes, since similar results were obtainable with cells from donors rendered tolerant to the test antigen.

In contrast to these observations, an immuno-depressive effect has also been ascribed to the GVHR, sometimes in the same studies which demonstrate its facilitatory qualities (4, 13, 26, 46). For this effect to be observed, the test antigen must usually be given some time after the third day of GVH activity. Davis et al (13) found that the response to an antigen decreased steadily as the interval between inoculation of the graft and subsequent immunization was extended from three to ten days. When the graft and the antigen were given on the same day, a boost in the response to antigen was seen. Immunization prior to initiation of the GVHR produced no discernible effect. Blaese et al (4), using a very strong GVH, showed no impairment of the immune response when the antigen was given prior to the graft; moderate depression if given on the same day; and virtual elimination of the humoral response when the antigen was given one day or more after the GVHR had commenced.

The late immuno-depressive effect of a GVHR is illustrated in Expt's. 1 and 2 of the present study, being seen in the normal group in one case and in the competition group in the other. The mechanism for this effect has not been clearly established. Möller (46) felt that it could not be attributed to a reduction in the number of antigen-sensitive cells, but was rather due to a humoral immunosuppressive factor released by cells of donor and/or host origin during the GVHR. Evidence for the existence

of such a factor was already discussed on pages 16 - 19 in relation to a possible mechanism for antigenic competition.

The stimulation seen during the early stages of a GVHR could similarly be attributed to a humoral substance having the opposite effect. Some investigators have already postulated the existence of such a substance, and linked it to the thymus (48, 49).

An immune response could thus be envisioned as the sum total of stimulatory and inhibitory influences which are acting in unison and whose relative proportions are changing over time. This concept seems to be supported (although somewhat inadvertantly) by the work of Lawrence and Simonsen (30), who originally set out to show something quite different. These workers initiated a GVHR in lethally irradiated mice, then administered a test antigen at varying intervals afterwards. Any response to the antigen had to be due to the grafted cells, since the host's own immunocompetent cells had been destroyed. The purpose of the experiment was to create a form of antigenic competition, in which the response to the test antigen would be depressed by previous exposure of the responding cells to another antigen -- the host itself. These results were indeed obtained when the test antigen was administered seven to ten days after initiation of the GVHR. However, if the interval was shortened to three days, exactly the opposite effect was observed -- a statistically significant boosting of the response to the test antigen.

Although the authors chose to explain their findings in

other terms, the results are certainly interpretable on the basis of a milieu being created by the GVHR, in which stimulatory influences predominate initially, and suppressive influences later on. The outcome of an antigenic challenge would then depend upon the particular "atmosphere" which prevailed during the afferent and/or efferent phases of the immune response.

Early facilitation by a mild GVH in non-preimmunized mice can account, at least in part, for the apparent accentuation of antigenic competition which appeared initially in each of the present experiments. The Index of Competition is lowered (indicating greater competition) by any widening of the difference between control group and competition group titers. This may come about in a number of ways: If the response in the control group is elevated by GVHR (as in Expt's. 1, 2, and 3), then a concomitant depression (Expt's. 1, 2), constancy, or less marked elevation (Expt. 3) in the competition group will seem to magnify the competitive effect (see Fig. 3a, b, c). Similarly, if the response in the control group is depressed by GVHR, then a more prominent depression in the competition group will also widen the difference (Fig. 3d, Expt. 4).

Analysis of these early events, as well as of those which occur later, is complicated by the extreme variability which is characteristic of the GVHR's effect upon immunological responsiveness. Under apparently identical conditions, for example, rats of one strain undergoing a GVHR may show an enhanced immunocompetence, while those of another strain may be immunologically depressed (46). The ability of a single agent to

stimulate or inhibit the immune response under different conditions is certainly well known (1, 18). The dosage of the agent, as well as the interval between its administration and subsequent antigenic challenge, are both instrumental in determining its effect. With respect to the GVHR, one might reasonably conclude that the host of parameters governing its own effects is by no means fully identified.

The late disappearance of competition in these experiments might also be mentioned, if only to caution against overinterpretation of this finding on the basis of GVH activity. Antigenic competition is normally a short-lived phenomenon, and its late disappearance would be expected in any case.

Much more worthy of consideration is the apparent ability of antigenic competition to modify the GVHR. This was often seen as an attenuation, in preimmunized mice, of the effects produced by the GVHR in normal mice. For example, if the response to SRBC was boosted by the GVH in normal mice, it would be boosted to a lesser extent in mice undergoing competition. Similarly, a depression of the response in a normal group would also be present, but to a less marked degree, in the corresponding competition group.

These findings lend themselves to a theory of "turnoff" which is consistent with that proposed for antigenic competition (pages 17 - 18). Such a theory assumes that an actively immunosuppressive milieu is created shortly after exposure to an antigenic stimulus, and it is this which transiently impairs the ability of the organism to respond to a subsequently

administered antigen. The milieu would come about through an active turnoff effect exerted by certain T-cells upon other T-cells and/or B-cells.

If this is used as a model, then the tendency of preimmunization to temper a GVHR could be explained in terms of a "turning off" of the grafted cells upon their introduction into the suppressive environment within the host. This environment would have been created by the previous immunization of the host and subsequent activation of his suppressor T-cells.

Studies have already shown that a GVHR may be attenuated by preimmunization of the donor with various antigens (32, 37). In this case, one might argue that the grafted cells had somehow been permanently altered by their exposure to turn-off within the donor, so that they were no longer capable of reacting normally to the stimulus provided by an allogeneic host. Alternatively, one could assume that the cells remained turned off for only a brief time following their removal from the host, but that the effect had not yet worn off at the time of initial contact with the host's tissue antigens. This would necessitate the further assumption that non-responsiveness to an antigen during initial contact precludes normal responsiveness to that antigen later on, after the effects of suppression have worn off. One piece of evidence against the latter view is the late elevation of the immune response which occurred in preimmunized mice undergoing a GVHR in Expt's. 1 and 3.

Conceivably, the present concept -- immunosuppression resulting from preimmunization -- could find application in a num-

ber of clinical areas, most notably with regard to human organ transplantation. According to the model, pretreatment with large doses of a benign antigen could mitigate some of the familiar rejection phenomena which follow the confrontation of the immune system with imperfectly matched tissue. Of course, there is little doubt that the actual mechanisms involved in the process are enormously more subtle than those depicted in the current representation. Successful clinical use of this principle would certainly have to be founded upon a more complete understanding of these subtleties.

IX. Summary and Conclusions

Antigenic competition was studied in normal mice and in mice inoculated with allogeneic thymocytes at the time of the second immunization. The following points were observed:

1. In normal (non-preimmunized) mice, the humoral antibody response was usually boosted during the early stages of a GVHR, and was variably affected during the later stages.

2. Antigenic competition appeared to be intensified by an early GVHR. This came about by:

   a) Depression of the antibody response in the competition groups and enhancement of the response in non-preimmunized mice (Expt's. 1, 3).

   b) Elevation of the response in both groups, but less so in the competition group (Expt. 2).

   c) Depression of the response in both groups, but more so in the competition group (Expt. 4).

3. The effects of a GVHR upon the immune response were usually mitigated or reversed by the presence of antigenic competition. Occasionally, however, they were intensified.

The results are discussed in terms of a fluctuating balance between immunosuppressive and immuno-stimulatory influ-

ences exerted by T-cells upon other T- and/or B-cells in response to antigenic provocation. Such influences could be mediated through the production of humoral substances by "stimulator" or "suppressor" T-cells. Evidence from other sources which is felt to be consistent with this view is presented and discussed.

X. Addendum

Since the conclusion of this study, Menkes, Hencin and Gershon (unpublished) have studied the mitotic activity of thymocytes grafted into preimmunized allogeneic hosts. Their results show that the GVHR, measured in terms of donor cell mitosis, is significantly reduced in the presence of antigenic competition. This is certainly consistent with some of the present observations. However, it does not explain accentuation or actual reversal of GVH effect by competition. Once again, the complexity of interplay between stimulatory and inhibitory influences must be invoked, not to obviate any further explanation, but rather to emphasize the necessity for more precise characterization of the cellular and humoral events surrounding both antigenic competition and the graft-versus-host phenomenon.

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GVHR	Antigenic Competition: Present	Antigenic Competition: Absent
Absent	Group 1	Group 2
Present	Group 3	Group 4

Fig. 1

		Experiment 1		Experiment 2	
		Competition	No Competition	Competition	No Competition
Day 4	GVH	0.80 ± 0.50	9.20 ± 1.02	1.20 ± 0.20	6.20 ± 0.37
	No GVH	2.00 ± 1.29	4.00 ± 1.22	0.80 ± 0.37	4.60 ± 1.50
Day 8	GVH	6.00 ± 0.84	9.20 ± 0.80	7.00 ± 1.38	12.60 ± 0.87
	No GVH	5.50 ± 0.92	8.25 ± 0.25	9.80 ± 1.46	12.60 ± 0.68
Day 15	GVH	9.60 ± 0.51	7.20 ± 0.80	9.60 ± 1.08	12.80 ± 0.80
	No GVH	8.00 ± 0.37	8.50 ± 0.65	11.80 ± 0.58	10.80 ± 0.97
Day 22	GVH	8.80 ± 0.58	7.00 ± 1.14	9.60 ± 1.08	9.80 ± 1.80
	No GVH	7.00 ± 0.52	7.50 ± 1.04	10.20 ± 1.20	7.40 ± 0.93

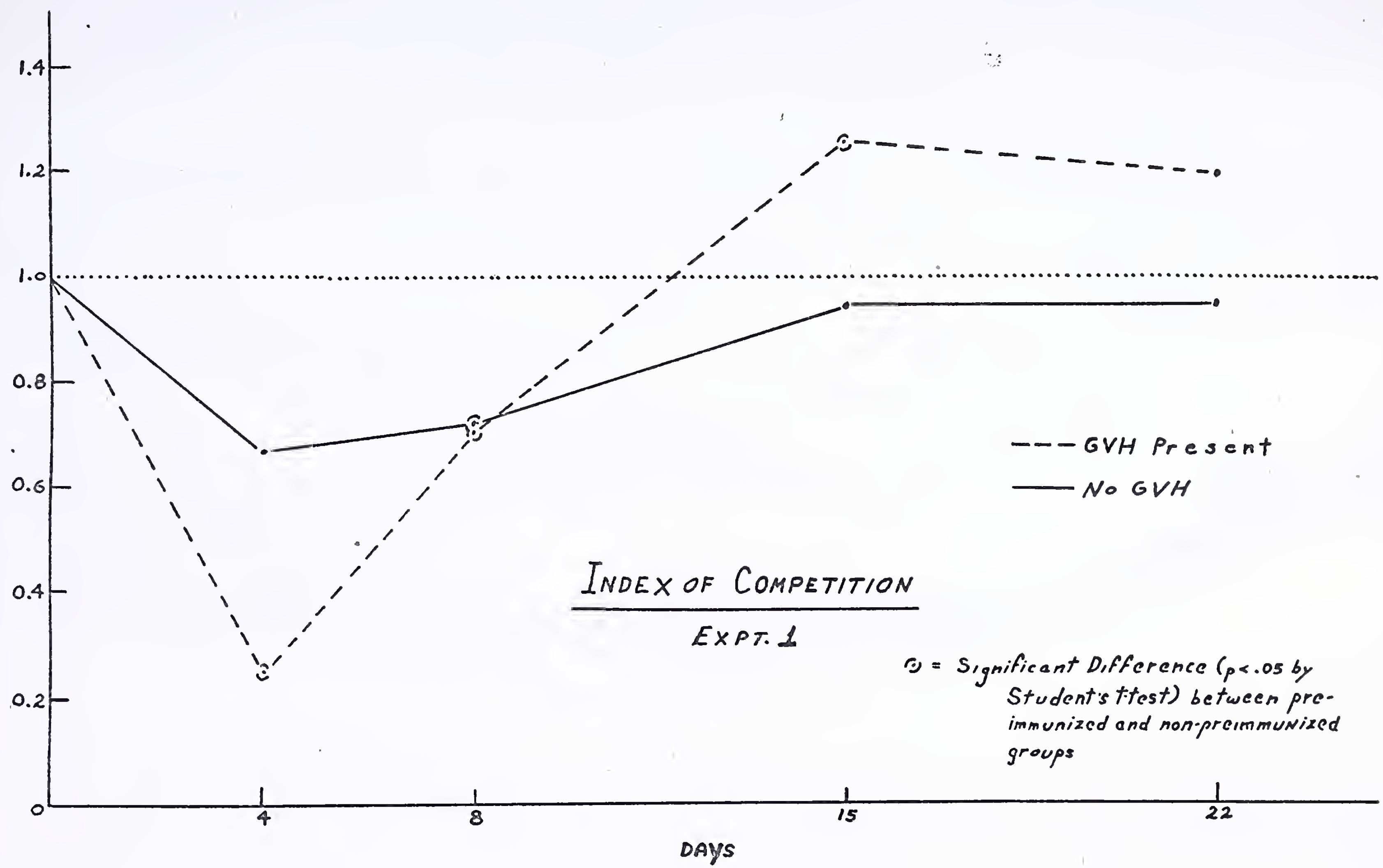
Table I a

Anti-SRBC Mean Titers ± S.E.
Expt's. 1 and 2

		Experiment 3		Experiment 4	
		Competition	No Competition	Competition	No Competition
Day 4	GVH	0.60 ± 0.68	2.60 ± 0.24	-0.83 ± 0.17	2.71 ± 1.11
	No GVH	1.00 ± 0.44	2.20 ± 0.73	-0.14 ± 0.55	3.14 ± 0.77
Day 8	GVH	7.00 ± 0.41	7.20 ± 0.49	7.60 ± 0.25	8.14 ± 0.26
	No GVH	6.00 ± 0.55	7.60 ± 0.24	7.16 ± 0.31	7.29 ± 0.29
Day 15	GVH	9.25 ± 0.48	9.00 ± 0.45	8.40 ± 0.40	9.71 ± 0.29
	No GVH	7.00 ± 0.32	8.60 ± 0.51	8.33 ± 0.33	9.33 ± 0.33
Day 22	GVH	8.00 ± 0.00	7.80 ± 0.20	6.20 ± 0.58	6.86 ± 0.59
	No GVH	6.20 ± 0.37	7.00 ± 0.32	6.33 ± 0.49	7.00 ± 0.37

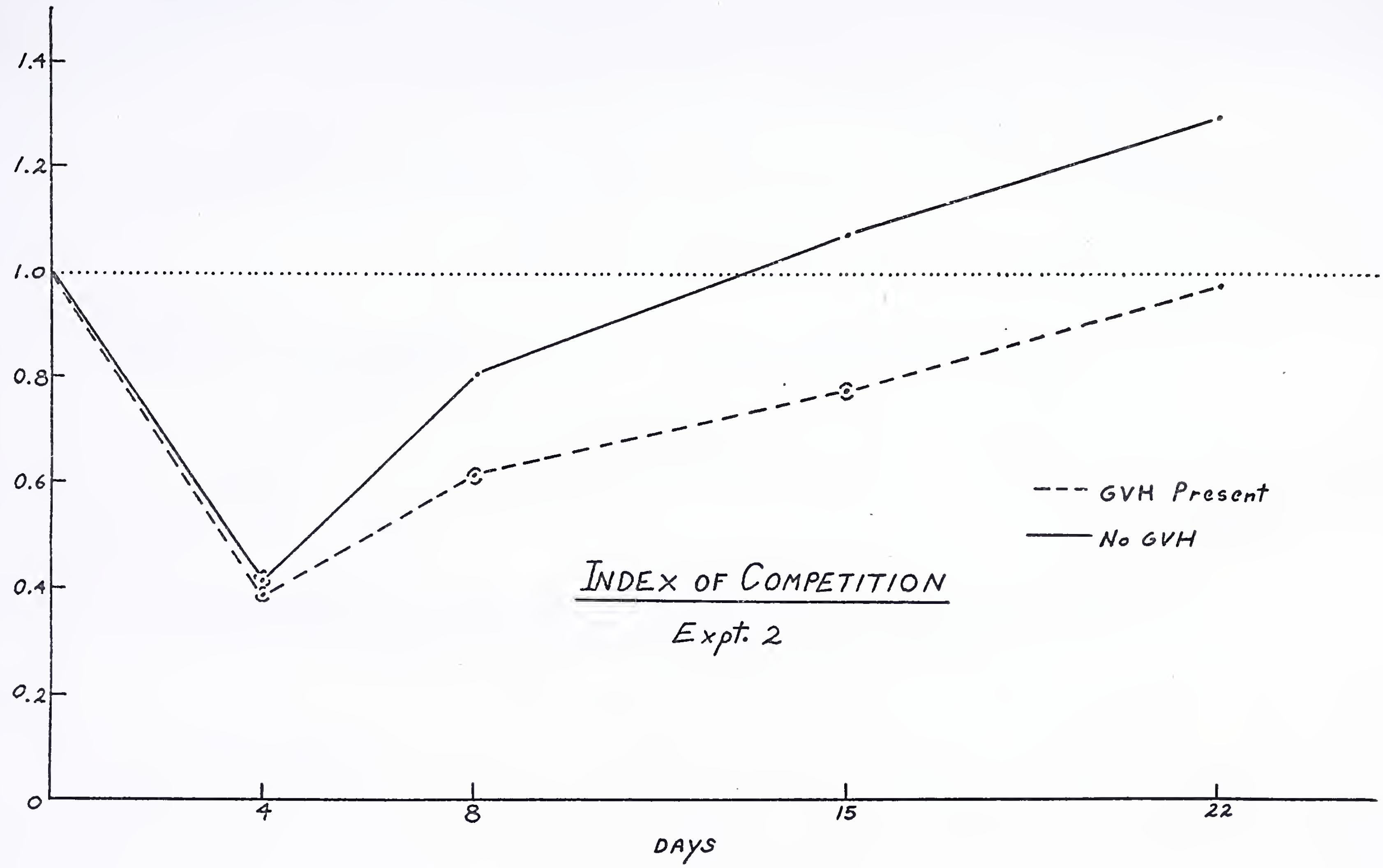
Table I b

Anti-SRBC Mean Titers ± S.E.
Expt's. 3 and 4



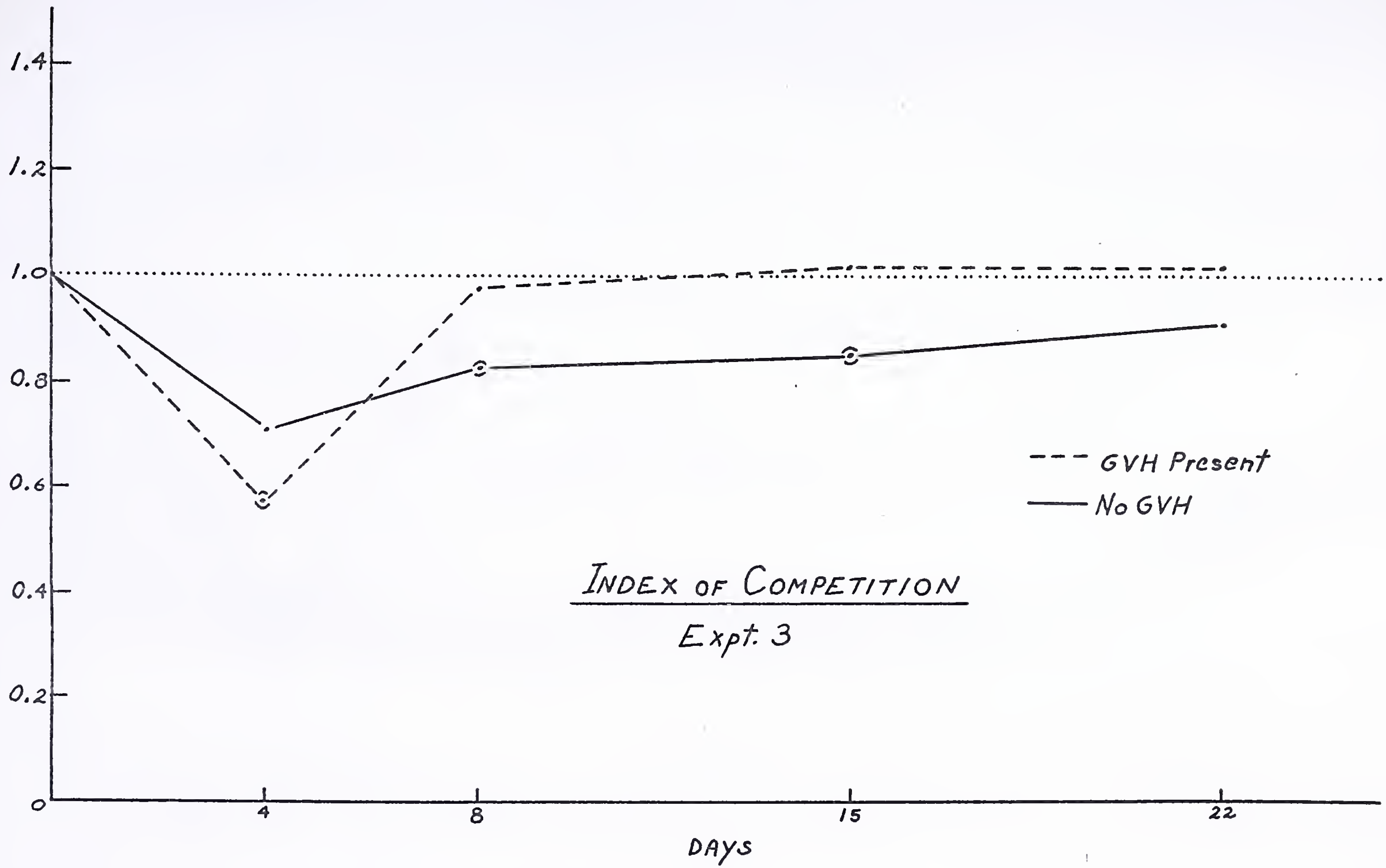
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
4
8
15
22
DAYS
--- GVH Present
— No GVH
INDEX OF COMPETITION
EXPT. 1
= Significant Difference (p<.05 by Student's t-test) between pre-immunized and non-preimmunized groups

Fig. 2a



Index of Competition
Expt. 2
GVH Present
No GVH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
4
8
15
22
DAYS

Fig. 2b



1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
4
8
15
22
DAYS
--- GVH Present
— No GVH
INDEX OF COMPETITION
Expt. 3

Fig. 2c

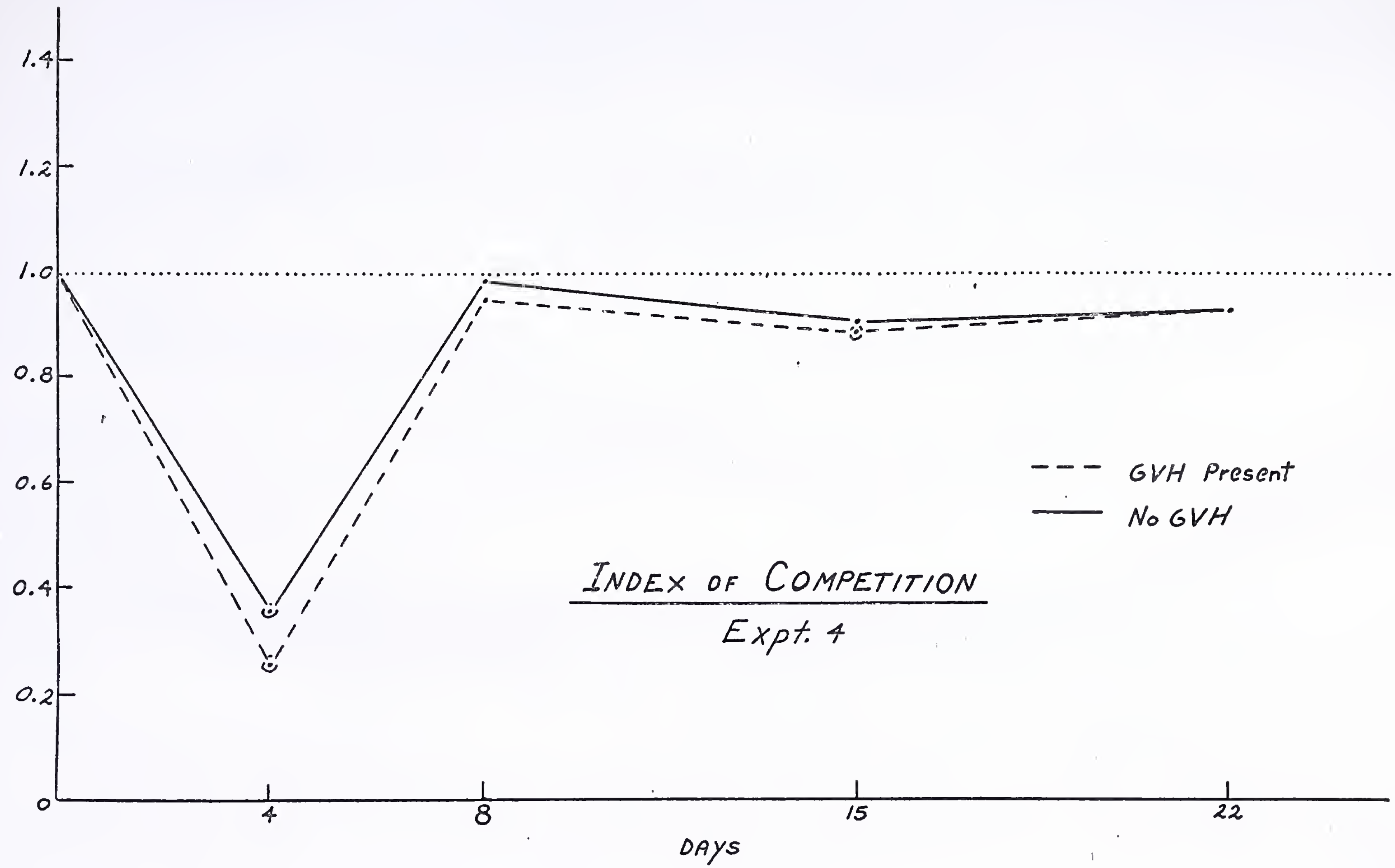
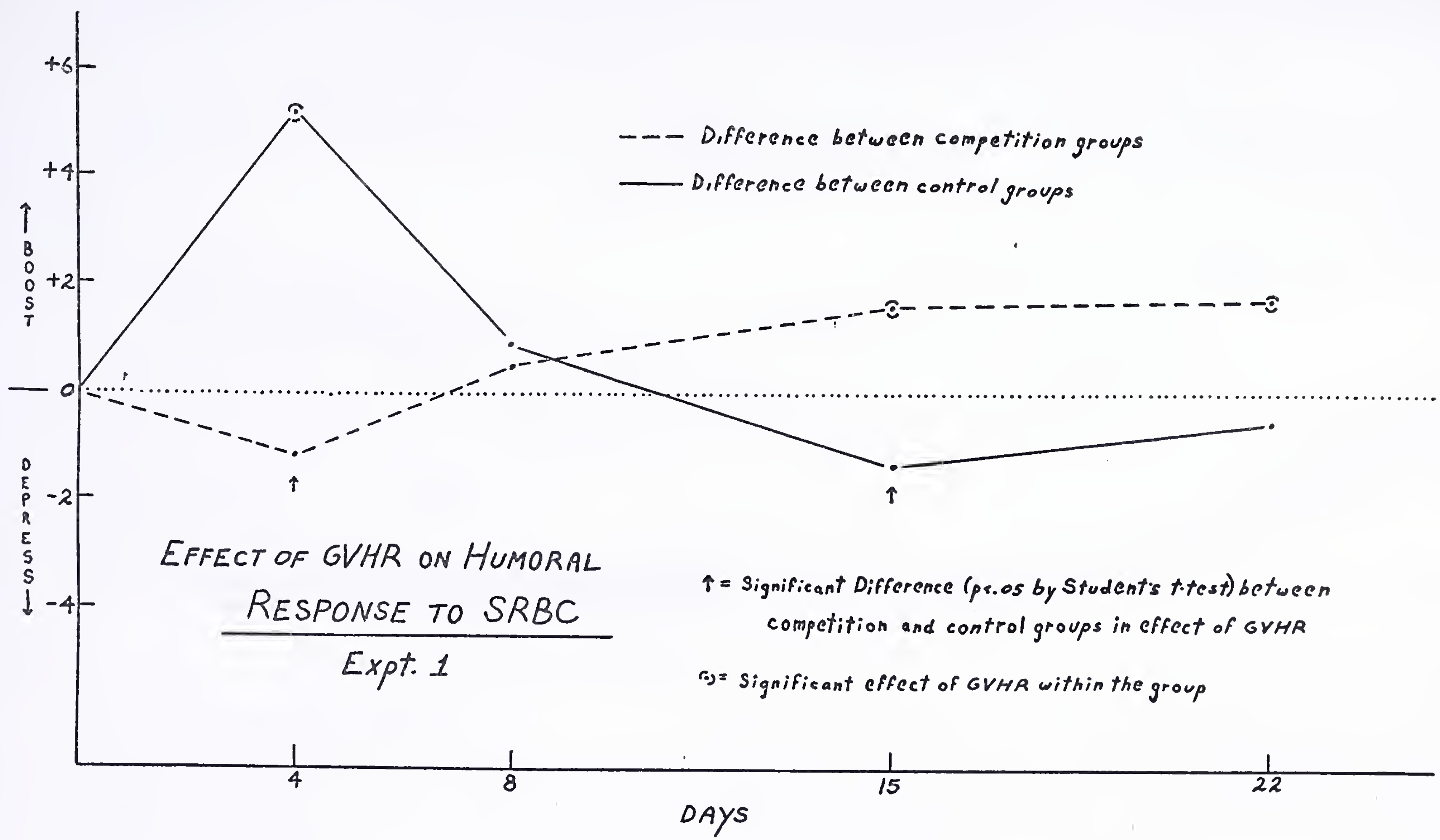
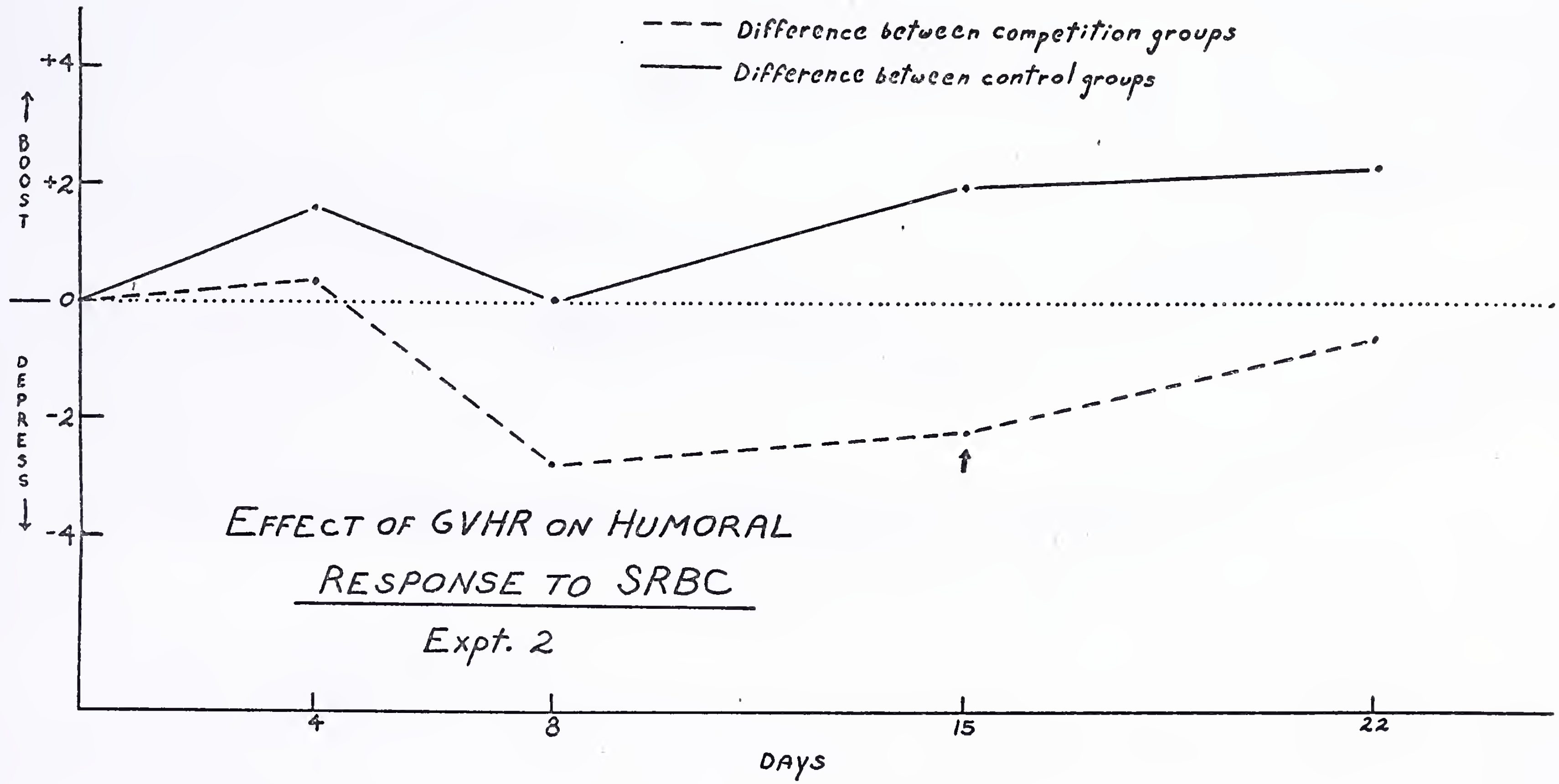

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
4
8
15
22
DAYS
GVH Present
No GVH
INDEX OF COMPETITION
Expt. 4

Fig. 2d



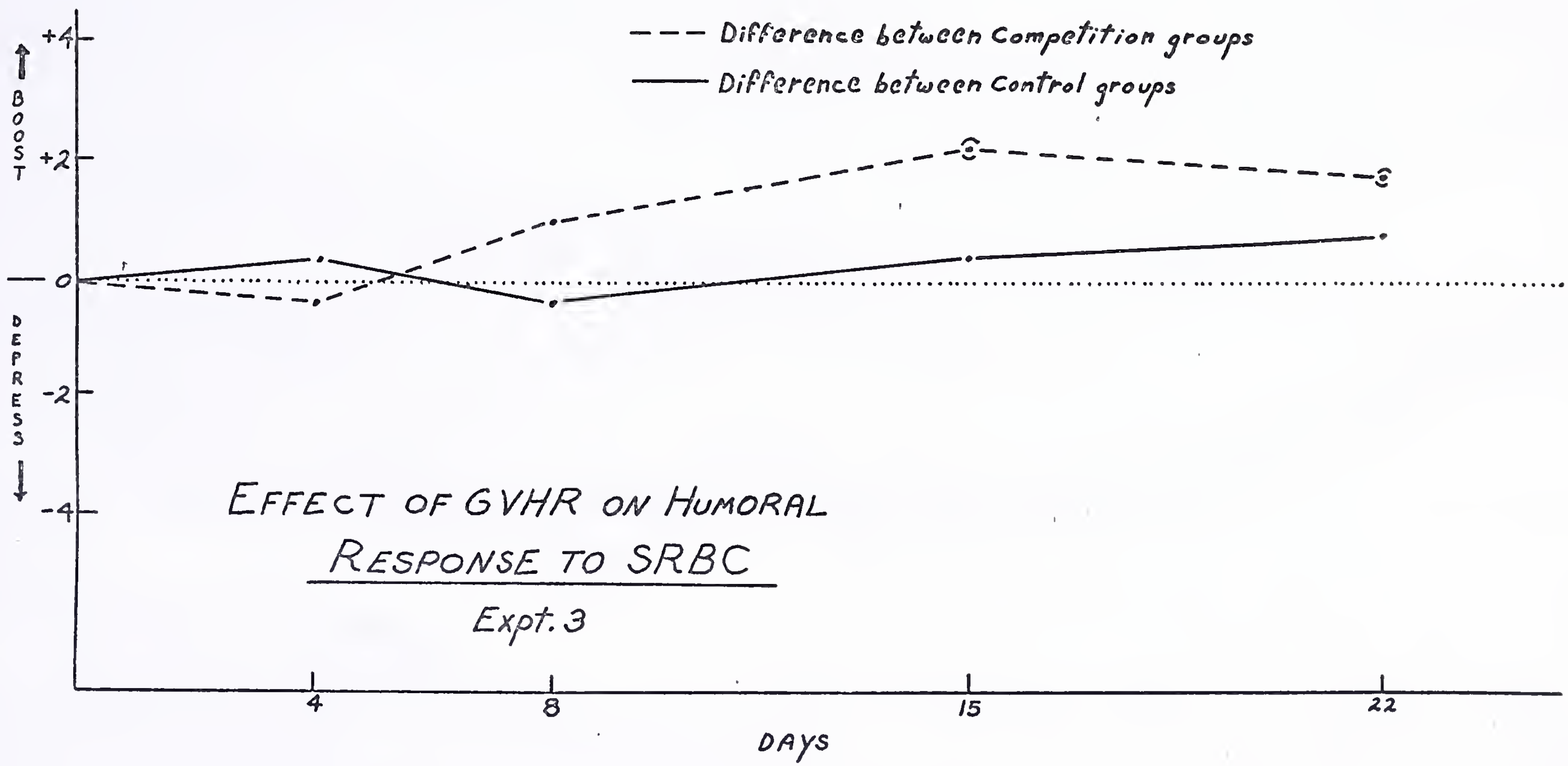
+6
+4
+2
0
-2
-4
BOOST
DEPRESS
--- Difference between competition groups
— Difference between control groups
EFFECT OF GVHR ON HUMORAL RESPONSE TO SRBC
Expt. 1
↑ = Significant Difference (p≤.05 by Student's t-test) between competition and control groups in effect of GVHR
(·) = Significant effect of GVHR within the group
4
8
15
22
DAYS

Fig. 3a



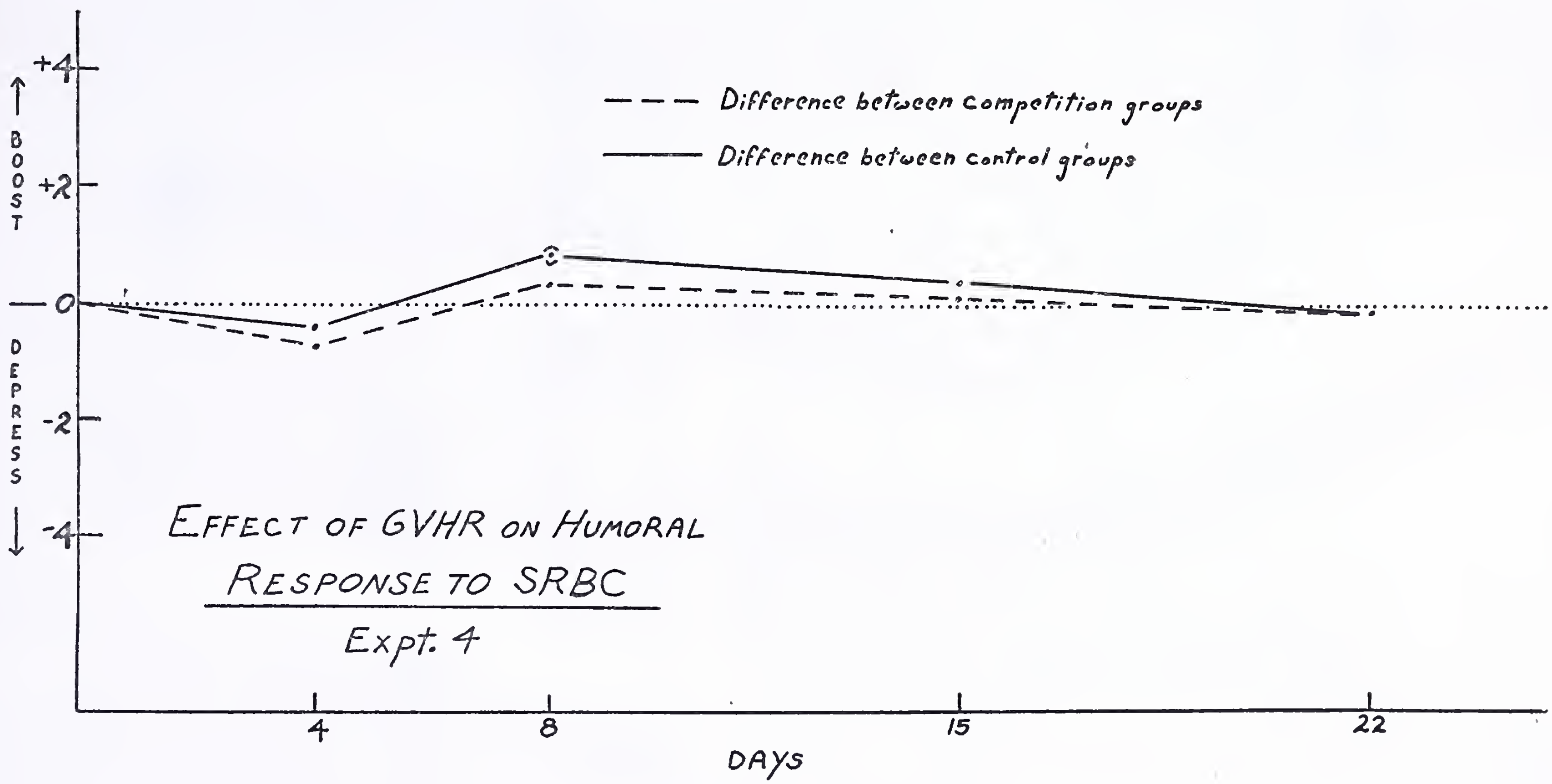
Difference between competition groups
Difference between control groups
+4
BOOST
+2
0
-2
DEPRESS
-4
EFFECT OF GVHR ON HUMORAL
RESPONSE TO SRBC
Expt. 2
4
8
15
22
DAYS

Fig. 3b



Difference between Competition groups
Difference between Control groups
BOOST
DEPRESS
+4
+2
0
-2
-4
EFFECT OF GVHR ON HUMORAL
RESPONSE TO SRBC
Expt. 3
4
8
15
22
DAYS

Fig. 3c



Difference between competition groups
Difference between control groups
BOOST
DEPRESS
+4
+2
0
-2
-4
EFFECT OF GVHR ON HUMORAL
RESPONSE TO SRBC
Expt. 4
4
8
15
22
DAYS

Fig. 3d

EXPERIMENT	DAY 4	DAY 8	DAY 15	DAY 22
1	R (p<.05)	Att	R (p<.05)	R
2	Att	Acc	R (p<.05)	R
3	R	R	Acc	Acc
4	Acc	Att	Att	Att

EFFECT OF ANTIGENIC COMPETITION UPON THE MANIFESTATIONS OF GVH

Acc = Accentuation of GVH effect
Att = Attenuation " " "
R = Reversal " " "

Fig. 4